527 Rec'd PCT/PTO 07 NOV 2000

FORM PTO-1390 (REV. 1-98) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER	U S. APPLICATION NO (If known, see 37 CFR 1 5
MSKP039	09/674975

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US99/10065	07 May 1999	08 May 1998

TITLE OF INVENTION
COMPOSITIONS AND METHODS FOR ACTIVE VACCINATION

APPLICANT(S) FOR DO/EO/US
Agus, et al.

EL556132244US

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 US.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 US.C. 371.
3. ☒ This express request to begin national examination procedures (35 US.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C371(b) and PCT Articles 22 and 39(1).

☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

☒ A copy of the International Application as filed (35 U.S.C.371(c)(2))
- a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
- b. ☐ has been transmitted by the International Bureau.
- c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).

☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

☒ Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
- a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau)
- b. ☐ have been transmitted by the International Bureau.
- c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
- d. ☒ have not been made and will not be made

☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C.371 (c)(3)).

☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

☐ A translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1 98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter
16. ☐ Other items or information:

Express Mail Cert.
Exp. Mail No.: EL556132244US
Date Mailed: 07 NOVEMBER 2000
Name: SUSAN HOWARD
Sign: Susan Howard

528 Rec'd PCT/PTO 07 NOV 2000

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/674975	PCT/US99/10065	MSKP039

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1 492 (a) (1) - (5)):	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1070.00	
International preliminary examination fee (37 CFR 1 482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... $930.00	
International preliminary examination fee (37 CFR 1 482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $790.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... $720.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $98.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 710.00
charge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 nths from the earliest claimed priority date (37 CFR 1.492(e)).	$

LAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
tal claims	20 - 20 =	0	x $22.00	$
pendent claims	4 - 3 =	1	x $82.00	$ 82.00
LTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$
TOTAL OF ABOVE CALCULATIONS =				$ 792.00
luction of 1/2 for filing by small entity, ifapplicable. A Small Entity Statement, t also be filed (Note 37 CFR 1.9, 1.27, 1.28). ☒ Applicant claims small entity status. See 37 CFR 1.27 +				$ - 396.00
SUBTOTAL =				$ 396.00
cessing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 ths from the earliest claimed priority date (37 CFR 1.492(f)).				$
TOTAL NATIONAL FEE =				$ 396.00
for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be mpanied by an appropriate cover sheet (37 CFR 3.28, 3 31). $40.00 per property +				$
TOTAL FEES ENCLOSED =				$ 396.00
			Amount to be refunded:	$
			charged:	$

a. ☒ A check in the amount of $ 396. to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 15-0610 A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

021121

PATENT TRADEMARK OFFICE

Marina T Larson
SIGNATURE

Marina T. Larson
NAME

32,038
REGISTRATION NUMBER

COMPOSITIONS AND METHODS FOR ACTIVE VACCINATION

BACKGROUND OF THE INVENTION

This application relates to an active vaccine approach to the treatment of cancer and other diseases. The approach is applicable to a number of cancers and diseases, although a preferred embodiment provides an active vaccine for treatment of B cell Non-Hodgkin's Lymphoma (NHL).

NHL is characterized by a clonal proliferation of malignant B cells. The treatment of NHL across a broad spectrum of patients remains a challenge, although numerous therapeutic approaches have been proposed and tried.

The most common therapeutic approach being used today is chemotherapy. While chemotherapy is effective for some period of time in most patients, a significant percentage of patients are not cured and experience a relapse.

Treatments have been proposed based on anti-idiotype therapy. In anti-idiotype therapy, a cell surface molecule which is expressed by malignant cells but not by normal cells is used to create patient-specific antibodies which are then administered to the patient. See, Miller, et al., *New Engl. J. Med.* 306: 517-522 (1982). Autologous patient-derived idiotype proteins have also been conjugated with keyhole limpet hemocyanin to produce a vaccine which has demonstrated efficacy and can elicit B and T cell immune responses. Kwak et al., *New Engl. J. Med.* 327: 1209-1215 (1992). Hybridoma-derived idiotype was co-cultured with patient-derived dendritic cells which acted as antigen presenters upon re-infusion into the patient and showed clinical efficacy. Hsu et al., *Nature Medicine* 2: 52-58 (1996). Idiotypic vaccines made in lipid-based carriers are disclosed in International Patent Publication WO98/14170.

Treatments have also been proposed using antibodies directed to CD20, a transmembrane protein that is expressed by both normal and malignant B-cells during parts of the B cell development cycle. Using single-dose infusions with anti-CD20 monoclonal antibodies, partial or minor tumor regressions were observed in 6 of 15 patients in a Phase I clinical study. Maloney et al., *Blood* 84: 2457-2466 (1994). In Phase II studies, 17 of 37 patients showed complete or partial remissions. In December 1997, the FDA approved the

first antibody-based therapy for NHL. Rituximab (Ritvaxan, IDEC/Genentech) is a chimeric human/murine antibody approved for the treatment of patients with relapsed or refractory low-grade or follicular $CD20^+$ B cell NHL. Maloney et al., *Blood* 90: 2188-2195 (1997).

Combinations of chemotherapy and anti-CD20 therapy have been reported as having better therapeutic efficacy, with 11 of 11 patients showing complete or partial remission. Czuczman et al., Abstract 53, *Ann. Oncol.* 7, Supp. 1: 56 (1996).

While therapeutic regimens using anti-CD20 concepts are potentially effective, all of these therapies have the drawback of being passive therapies, i.e., they do not directly involve the immune system of the patient. Thus, these therapies may require the continued administration of the therapeutic agent for efficacy and do not provide any long-term protection against recurrence. In addition, the passive therapy is monoclonal in nature, therefore escape is possible. It would therefore be desirable to have an active therapy, that is a therapeutic agent which when administered to the patient stimulates an immune response against CD20 found in B-cells.

It is an object of the present invention to provide such a therapy. It is a further object of the invention to provide an active polyclonal therapy that is difficult to evade.

SUMMARY OF THE INVENTION

In accordance with the present invention, NHL is treated, not by administration of an anti-CD20 monoclonal antibody, but by the administration of CD20 itself, or an immunogenic fragment of the extracellular portion thereof, coupled to or administered with an antigenic carrier moiety such as keyhole limpet hemocyanin (KLH). This results in the stimulation of the production of polyclonal antibodies against CD20 (or an immunogenic fragment thereof) which has the affect of reducing the number of B-cells, including malignant B-cells. Thus, the invention provides an active vaccine. The same approach can be used for therapeutics for other diseases and conditions in which target cells possess a transmembrane protein, and is particularly applicable to those diseases and conditions for which administration of antibodies to transmembrane proteins or peptides (i.e., passive therapy) have been shown to provide therapeutic benefits, and especially in the

situations where the target is also capable of transducing or receiving a signal important for cell growth or function. This would include, for example, Her2/neu, VEGF receptor, epidermal growth factor receptor, the CD19 molecule, interleukin-2-receptor, interleukin-4-receptor, and the P-glycoprotein, also known as the multidrug-resistance protein.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A and B show ELISA results for formation of antibodies to human and mouse CD20 in vaccinated mice;

Figs. 2A and B shows results for binding of control B1 antibodies or antibodies in plasma from a mouse treated with human CD20-KLH conjugate with Raji B NHL cells;

Fig. 3 shows $CP19^+$ B cell levels in mice treated with human or mouse CD20-KLH conjugate;

Fig. 4 shows the domain structure of human Her2;

Fig. 5 shows the domain structure of human EGFR;

Figs. 6A-D shows the cross-reactivity of antibodies generated in response to human or mouse CD20 fragments;

Figs. 7A-D show the importance of carrier protein and adjuvant in generating an immune response;

Figs. 8A-D shows the immune response generated using different adjuvants; and

Figs. 9A-I shows $CP19^+$ B cell levels in mice treated with human or mouse CD20-KLH conjugate.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an active vaccine therapy which can be used in the treatment of a variety of cancers and related conditions in which it is desirable to bring about the death of a target group of cells. Conventionally, immunotherapies targeting cells have sought to obtain a cellular immune response (T-cells that recognize the target cells), since a humoral immune response (antibodies that recognize the target cells) alone is not

deemed sufficient to achieve the desired result of cell death. The present invention departs from this conventional wisdom, and effectively utilizes a humoral immune response against the target cells to provide therapeutic benefit. The targets for therapy include cell surface proteins that when bound by a ligand signal to the cell. The vaccine induced antibody response will mimic ligand binding and cause similar signaling events which can imitate the process of programmed cell death (apoptosis) or halt the cell from growing or change the cancer cell's sensitivity to chemotherapy.

By way of example, the invention is suitably employed in the treatment of NHL and other B cell diseases such as chronic lymphocytic leukemia, auto-immune disorders and B-cell regulatory disorders. In accordance with this embodiment of the invention, a peptide antigen is prepared which contains at least an immunogenic portion of the extracellular domain of CD20 coupled to or administered with an antigenic carrier protein. The CD20 component of the peptide antigen may be syngeneic or it may be xenogeneic. Thus, for example, human patients may be treated with a peptide vaccine containing a human or a mouse CD20-fragment. There is evidence that strong immune responses can be elicited against xenogeneic proteins. Naftzger et al., *Proc. Natl. Acad. Sci. (USA)* 93: 14809-14814 (1996); International Patent Application PCT/US97/22669, filed December 10 1997, incorporated herein by reference. A suitable fragment is the 44 amino acid peptide spanning amino acids 136 to 179 of the sequence of mouse or human CD20. (Seq. ID Nos. 1 and 2) Other immunogenic fragments derived from the extracellular domain of CD20, or the entire CD20 molecule may also be used. Seq. ID. Nos. 3 and 4 shows the nucleic acid and amino acid sequences, respectively, of exon VI (the extracellular domain) of human CD20 as reported by Tedder et al., *J. Immunol.* 142: 2560-2568 (1989).

As used in the specification and claims hereof, an "immunogenic fragment" is a molecule which includes at least a portion of the extracellular domain of a transmembrane protein to direct and immunological response to that transmembrane protein when the immunogenic fragment is coupled to or administered with an antigenic carrier protein effective to break tolerance and administered with an adjuvant. It is not required that the immunogenic fragment alone be effective to stimulate an immune response, although such stimulation would not take a given fragment outside the scope of the present invention.

A preferred antigenic carrier protein is keyhole limpet hemocyanin which can be coupled to peptides using techniques described in Pierce Catalog Protocol. Other antigenic carrier proteins which can be used to break tolerance might be used in the invention include immunoglobulins, tuberculin, tetanus toxin and others well known in the art.

The peptide antigen containing the CD20 component and the antigenic carrier protein is formulated with a pharmaceutically acceptable adjuvant in a liquid carrier and administered to a patient suffering from NHL or another B cell disease. The composition will generally be administered by injection, for example, intramuscular, subcutaneous or intradermal injection, but might also be administered by way of a DNA vaccine (See US Patent No. 5,580,859, incorporated herein by reference) or a viral vaccine, or after mixing with antigen presenting cells (APC's) such as dendritic cells, *ex vivo*. Alternatively, the antigen may be administered without adjuvant by injection into a host prepared by prior or simultaneous injection of an immune adjuvant. Specific amounts to be administered to a patient can be determined by monitoring the titer of anti-CD20 antibodies developed by the patient, or by an average group of patients using well-known technology.

When a peptide of the extracellular domain of human or mouse CD20 is coupled to KLH and administered with an adjuvant to mice, antibodies which react with CD20 are found in plasma. (Figs. 1A and B) These antibodies bind to Raji cells, a human lymphoma cell line, indicating the ability to bind to a cell expressing CD20. (Figs. 2A and B). Moreover, the number of $CD19^+$ B cells present in mice injected with either of the two CD20-KLH conjugates declines substantially (~30% decrease relative to controls). (Figs. 3 and 9). The assay used to quantitate B cell depletion detects CD19 which is also expressed on immature B cells that are $CD20^-$. Thus, the 30% depletion actually underestimates the efficacy of the vaccine against $CD20^+$ B cells. .

Antibodies generated in mice after vaccination with human or mouse-derived CD20 fragments are specific for the peptides used, yet are capable of inducing immunity to the corresponding peptide from other species (Figs. 6A-D). Studies showed that in most instances the peptide, carrier protein and adjuvant are all needed for optimal response, although some responses were detected using less than all of the components. (Figs. 7A-D).

Several different adjuvants were also tested, and QS21 was found to be the most effective of those tested. (Fig. 8A-D).

While not intending to be bound by any particular mechanism, it is believed that the vaccines of the present invention are effective via at least two pathways. First, the generation of a humoral immune response to CD20 is effective to some extent to reduce the numbers of B cells bearing CD20 antigen in a manner consistent with normal immunological response to a target antigen. In addition, however, because CD20 has a signaling function, the binding of antibody to the CD20 moiety activates this signaling function to trigger apoptotic cell death. Such stimulation of apoptosis has been demonstrated to occur *in vitro* following passive treatments with a chimeric anti-CD20 antibody. Maloney et al., *Blood* 88 (Supp. 1): 637a (1996).

It is also possible that T cell mediated effector mechanisms are involved in the immune response. As evidence of this, we illustrate in Table 1 the mouse and human peptide sequences capable of binding to the corresponding mouse and human histocompatability antigens. This information was derived from a search of the NIH Bioinformatics and Molecular Analysis Section HLA Binding Predictions database using the mouse and human CD20 amino acid sequences. (Parker et al., *J. Immunol.* 152: 163 (1994)).

While the method of the invention is illustrated here using CD20 or CD20-derived peptides as the antigen to target B cells, the invention is not limited to this embodiment. Rather, the inventions encompasses the use of vaccine compositions comprising an immunogenic portions of the extracellular domain of transmembrane protein or peptide, particularly a transmembrane protein or peptide having signaling function, coupled to or administered with an antigenic protein and/or adjuvant to break tolerance.

A non-limiting example of another transmembrane protein which can be used in whole or in part in the method of the invention is Her-2/neu. The Her-2/neu oncogene is a receptor-like tyrosine kinase that is expressed on the cell surface of a significant portion of solid tumors. It has been shown that patients with early stage breast cancer have a high titer of antibodies to Her-2/neu. Disis et al., *J. Clin. Oncol.* 15: 3363-3367 (19967). The amino acid sequence and domain structure of human Her-2/neu are shown in Seq. OD. No. 5 and Fig. 4, and isolation and expression of the extracellular domain has been disclosed.

International Patent Publication No. WO 90/14357, which is incorporated herein by reference. There is clinical data showing efficacy of monoclonal antibodies against Her-2-neu in the treatment of patients with Her-2/neu$^+$ tumors, and potential synergism with chemotherapy. Thus, in accordance with the present invention, a vaccine composition comprising at least an immunogenic portion of the extracellular domain of Her-2-neu (amino acids 22 to 652) coupled to or administered with an antigenic protein or peptide such a KLH can be used as a vaccine to provide the same therapeutic benefits using an active as opposed to a passive approach.

A further non-limiting example of a transmembrane protein which can be used in whole or in part in the method of the invention is epidermal growth factor receptor (EGFR). The amino acid sequence and domain structure of human EGFR are shown in Seq. ID. No. 6 and Fig. 5. There is significant data showing that antibodies to EGFR can have anti-tumor activity in breast and prostate cancer, as well as several head and neck tumors. Prewett et al., *J. Immunother. Emphasis Tumor Humoral* 19: 419-27 (1996). The mechanism by which antibody therapy against EGFR may be efficacious can be through the ability to down-regulate vascular endothelial growth factor production by tumor cells and thereby decrease angiogenesis. Petit et al., *Am. J. Pathol.* 151: 1523-30 (1997). In accordance with the present invention, a vaccine composition comprising at least an immunogenic portion of the extracellular domain of EGFR (amino acids 25 to 645) coupled to or administered with an antigenic protein or peptide such a KLH can be used as a vaccine to provide the same therapeutic benefits using an active as opposed to a passive approach. Preferred immunogenic peptides would be selected from regions not deleted in the various types of truncated EGFR mutants associated with some cancers.

A further non-limiting example of a transmembrane protein which can be used in whole or in part in the method of the invention is VEGF receptor. There are significant data showing that antibodies to VEGF receptor can inhibit angiogenesis and thereby halt tumor progression. In accordance with the present invention, a vaccine composition comprising at least an immunogenic portion of the extracellular domain of VEGF receptor coupled to or administered with an antigenic protein or peptide such a KLH can be used as a

vaccine to provide the same therapeutic benefits using an active as opposed to a passive approach.

Still a further non-limiting example of a transmembrane protein which can be used in whole or in part in the method of the invention is the IL-2 receptor. The IL-2 receptor is expressed on most T-cells malignancies, and there is a data showing that antibodies to the IL-2 receptor can be used in the treatment of T-cell malignancies and autoimmune disorders. In the present invention, a composition is made comprising at least an immunogenic portion of the extracellular domain of the IL-2 receptor (e.g., P55 or P75) , coupled to or administered with an antigenic carrier protein or peptide such as KLH. and used as a vaccine.

The vaccine compositions of invention can be used alone or in combination (concurrently or sequentially) with drugs or chemotherapy agents that provide therapeutic benefit for the condition being treated. In the case of NHL, suitable chemotherapy agents which can be used in combination with the CD20 based vaccine include alkylating agents, anthrocyclines, cis-platinum, fludarabine, corticosteroids and vinca alkaloids. These same chemotherapy agents which might be used in combination with other vaccine compositions for other forms of cancer.

EXAMPLE 1

44 amino acid fragments of the extracellular domains of humans and murine CD20 (amino acids 136-179, Seq. ID Nos. 1 and 2) were synthesized using a solid-phase FMOC peptide synthesizer and coupled to KLH using the methodology described in the Pierce Catalog Protocol. The peptide coupled to KLH was then prepared for injection by formulation with QS-21 adjuvant. Balb/c mice were injected according to one of the following protocols on days 1, 8, 15, 22 and 50 of the experiment:

A. Murine CD20 fragment-KLH with QS-21 adjuvant
B. Human CD20 fragment-KLH with QS-21 adjuvant
C. KLH with QS-21 adjuvant
D. QS-21 adjuvant

E. P190 (irrelevant protein) coupled to KLH with QS-21 adjuvant

F. B3A2 (irrelevant peptide) coupled to KLH with QS-21 adjuvant.

The animals were sacrificed on day 62 of the experiment.

Serum samples from the mice were diluted 1:200 and evaluated by BSA-blocked ELISA using goat-anti-mouse antibody conjugated to alkaline phosphatase for antibodies which bind to human CD20, mouse CD20 and KLH. As shown in Figs 1A and B, mice injected with human CD20 coupled to KLH (Fig. 1A) or mouse CD20 coupled to KLH (Fig. 1B) administration of xenogeneic antibody produced a significant polyclonal antibody response to both human and mouse CD20, while the response following administration of syngeneic antibody was principally limited to antibodies to the syngeneic form of CD20. Either xenogeneic or syngeneic peptide can therefore be used to generate an immune response.

To confirm the ability of the antibodies to bind to B cells, Raji cells (a form of human B-cell lymphoma that expresses CD20 on its surface) were blocked with human IgG, washed and then incubated for 30 minutes on ice with a 1:10 dilution of plasma from a mouse vaccinated with P-190-KLH control or huCD20-KLH. As a positive control, Raji cells were incubated with B1 antibody, or IgG2 as an isotypic negative control. After washing, the cells were incubated with goat-anti-mouse antibody, washed and fixed with 1% paraformaldehyde. Flow cytometry analysis was performed in a Becton-Dickinson FACScan. The results are shown in Figs 2A and 3B, wherein the shaded data set are the experimental data set and the outlined data set is the negative controls. As is apparent, there is a strong binding of mouse antibodies and Raji cells, comparable to that observed with B1 antibody .

EXAMPLE 2

To assess the number of B cells present in vaccinated mice, an evaluation was made of cells expressing CD19, a standard phenotypic marker for B cells. Spleens were harvested from the animals vaccinated in Example 1 and put into a single-cell suspension. After counting the total number of cells, the cells were stained with FITC-labeled anti-mouse CD19 and the samples were analyzed by flow cytometry with a FACScan. 10,000 events

were collected. The percentage of CD19 positive cells minus the control gate was multiplied by the total number of cells to determine the number of CD19 positive cells in mice treated with the mouse and human CD20 peptide conjugates, and the P190 irrelevant peptide conjugate control.

As shown in Fig. 3, the absolute number of CD19 positive cells was significantly reduced in mice treated with either of the CD20 peptide conjugates. The level of CD19 positive cells is a reflection of the number of CD20 positive B cells, and the number of immature $CD19^+$, $CD20^-$ B cells in the samples. The absolute number of $CD19^+$ B cells actually underestimates the therapeutic efficacy of the treatment for elimination of $CD20^+$ B cells, however, since CD19 is expressed on B cell progenitor cells before expression of CD20.

EXAMPLE 3

Mice were injected five times over two months with one of four treatment protocols as follows:

human CD20 (44 aa fragment)-KLH plus QS1

human CD20 (44 aa fragment)-KLH

human CD20 (44 aa fragment) plus QS21

KLH plus QS21

Blood was collected on week 9 for analysis by ELISA. Sera from the vaccinated mice were diluted 1:200 and incubated on BSA blocked plates coated with msCD20, huCD20, P190 or KLH. Secondary goat anti-mouse antibody conjugated to alkaline phosphatase was added, and the color change of p-nitrophenyl phosphate substrate was measured at 405 nm. The results are summarized in Figs. 7A-D. In most instances the peptide, carrier protein and adjuvant are all needed for optimal response, although some responses were detected using less than all of the components.

EXAMPLE 4

Mice were vaccinated according to the schedule of Example 3 using one of four treatment protocols: human CD20 (44 aa fragment)-KLH plus QS21 adjuvant, mouse

CD20 (44 amino acid fragment)-KLH plus QS21, P190 (irrelevant protein)-KLH +QS21 and KLH and QS21 alone. Mouse serum samples were evaluated by ELISA for the presence of antibodies reactive with msCD20, huCD20, P190 and KLH. The results are shown in Figs. 6A-D. Antibodies generated in mice after vaccination with human or mouse-derived CD20 fragments are specific for the peptides used, yet are capable of inducing immunity to the corresponding peptide from other species.

EXAMPLE 5

Mice were vaccinated five times over two months with huCD20 fragment-KLH conjugate with no adjuvant or in combination with one of three adjuvants: QS21, BCG or Alum. Serum samples from the vaccinated mice were tested by ELISA. The results are summarized in Figs. 8A-D. QS21 was found to be the most effective of those tested.

EXAMPLE 6

To confirm the observations of Example 2, nucleated spleen cells were recovered by centrifugation in a density gradient from mice vaccinated with a CD20-KLH conjugate (human or mouse) in the presence of QS21 adjuvant. 1 X 10^6 cells from each mouse were incubated with 2 μg of rat anti-mouse CD19 FITC-labeled antibody or with isotope-matched FITC labeled rat antibody. Cells were washed, fixed and analyzed with a Becton Dickinson FACScaliber cytometer. Figs 9A-C, D-F and G-I show the results for three exemplary mice of each vaccination group. The decrease in the peak reflecting levels of CD19 positive spleen cells in each of the mice is apparent.

Table 1

Species	Peptide Sequence	HLA Molecule	Score ($T_{1/2}$ of Dissociation of Molecule Containing this Subsequence)
human	NFIRaHTPYI	Kd	1600
human	FIRAHTPYI	Kd	48
human	FLKMeSLNFI	A_0201	21.2
mouse	HFLKmRRLEL	Kd	1600
mouse	IYDCePSNSS	Kd	60
mouse	LIQTSKPYV	A_0201	89.4
mouse	ELIQtSKPYV	A_0201	28.7

CLAIMS

1. A method for active vaccination against autologous cells expressing transmembrane proteins comprising administering to a patient a vaccine composition comprising at least an immunogenic portion of the extracellular domain of the transmembrane protein, or a xenogeneic homolog thereof, coupled to or administered with an carrier protein effective to break tolerance to the transmembrane protein and a pharmaceutically acceptable adjuvant.

2. The method of claim 1, wherein the transmembrane protein is selected from the group consisting of CD20, Her2-neu, VEGF receptor, epidermal growth factor receptor, the CD19 molecule, interleukin-2-receptor, interleukin-4-receptor, and the P-glycoprotein.

3. The method of claim 1, wherein the transmembrane protein is CD20.

4. The method of claim 1, wherein the vaccine composition comprises a peptide having the sequence given by Seq. ID No 1 or 2.

5. The method claim 1, wherein the carrier protein is keyhole limpet hemocyanin.

6. The method of claim 5, wherein the transmembrane protein is selected from the group consisting of CD20, Her2-neu, VEGF receptor, epidermal growth factor receptor, the CD19 molecule, interleukin-2-receptor, interleukin-4-receptor, and the P-glycoprotein.

7. The method of claim 5, wherein the transmembrane protein is CD20.

8. The method of claim 7, wherein the vaccine composition comprises a peptide having the sequence given by Seq. ID No 1 or 2.

9. A method for active vaccination against B cells expressing CD20 comprising administering to a patient a vaccine composition comprising at least an immunogenic portion of the extracellular domain of CD20, or a xenogeneic homolog thereof, coupled to or administered with an carrier protein effective to break tolerance to the transmembrane protein and a pharmaceutically acceptable adjuvant.

10. The method claim 9, wherein the carrier protein is keyhole limpet hemocyanin.

11. The method of claim 9, wherein the vaccine composition comprises a peptide having the sequence given by Seq. ID No 1 or 2.

12. A method for treatment of B cell non-Hodgkin's lymphoma, comprising administering to a patient suffering from B cell non-Hodgkin's lymphoma a vaccine composition comprising at least an immunogenic portion of the extracellular domain of CD20, or a xenogeneic homolog thereof, coupled to or administered with an carrier protein effective to break tolerance to the transmembrane protein and a pharmaceutically acceptable adjuvant.

13. A vaccine composition comprising at least an immunogenic portion of the extracellular domain of the transmembrane protein, or a xenogeneic homolog thereof, coupled to or administered with an carrier protein effective to break tolerance to the transmembrane protein and a pharmaceutically acceptable adjuvant.

14. The composition of claim 13, wherein the transmembrane protein is selected from the group consisting of CD20, Her2-neu, VEGF receptor, epidermal growth

factor receptor, the CD19 molecule, interleukin-2-receptor, interleukin-4-receptor, and the P-glycoprotein.

15. The composition of claim 13, wherein the transmembrane protein is CD20.

16. The composition of claim 15, wherein the vaccine composition comprises a peptide having the sequence given by Seq. ID No 1 or 2.

17. The composition of claim 13, wherein the carrier protein is keyhole limpet hemocyanin.

18. The composition of claim 17, wherein the transmembrane protein is selected from the group consisting of CD20, Her2-neu, VEGF receptor, epidermal growth factor receptor, the CD19 molecule, interleukin-2-receptor, interleukin-4-receptor, and the P-glycoprotein.

19. The composition of claim 17, wherein the transmembrane protein is CD20.

20. The composition of claim 19, wherein the vaccine composition comprises a peptide having the sequence given by Seq. ID No 1 or 2.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A01N 37/18

A1

(11) International Publication Number: WO 99/57981

(43) International Publication Date: 18 November 1999 (18.11.99)

(21) International Application Number: PCT/US99/10065

(22) International Filing Date: 7 May 1999 (07.05.99)

(30) Priority Data:
60/084,870 8 May 1998 (08.05.98) US

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(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: AGUS, David, B. [US/US]; 9 Pierrepont Street, Brooklyn, NY 11201 (US). SCHEINBERG, David [US/US]; 325 Central Park West, New York, NY 10025 (US). ROBERTS, Wendy [US/US]; 1233 York Avenue, New York, NY 10021 (US). ZELENETZ, Andrew, D. [US/US]; 31 Mohegan Road, Larchmont, NY 10538 (US).

(74) Agent: LARSON, Marina, T.; Oppedahl & Larson LLP, P.O. Box 5270, Frisco, CO 80443 (US).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: COMPOSITIONS AND METHODS FOR ACTIVE VACCINATION

(57) Abstract

Non–Hodgkin's lymphoma (NHL) is treated, not by administration of an anti–CD20 monoclonal antibody, but by the administration of CD20 itself, or an immunogenic fragment of the extracellular portion thereof, coupled to or administered with an antigenic carrier moiety such as keyhole limpet hemocyanin (KLH). This results in the stimulation of the production of polyclonal antibodies against CD20 (or an immunogenic fragment thereof) which has the effect of reducing the number of B–cells, including malignant B–cells, and thus provides an active vaccine. The same approach can be used for therapeutics for other diseases and conditions in which target cells possess a transmembrane protein, and is particularly applicable to those diseases and conditions for which administration of antibodies to transmembrane proteins or peptides (i.e., passive therapy) have been shown to provide therapeutic benefits, and especially in the situations where the target is also capable of transducing or receiving a signal important for cell growth or function. This would include, for example, Her2/neu, VEGF receptor, epidermal growth factor receptor, the CD19 molecule, interleukin–2–receptor, interleukin–4–receptor, and the P–glycoprotein, also known as the multidrug–resistance protein.

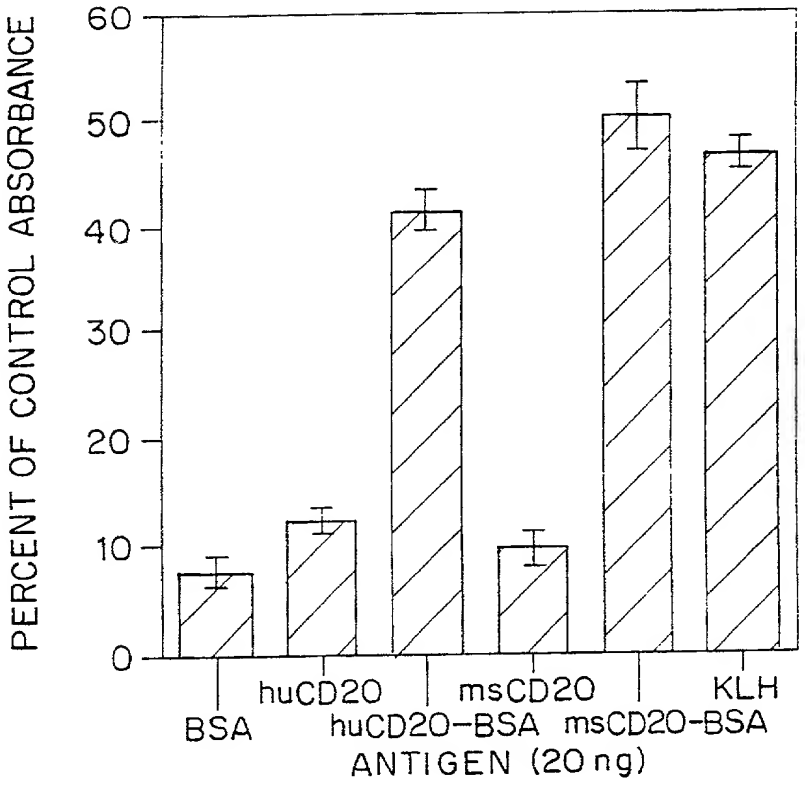

PERCENT OF CONTROL ABSORBANCE
60
50
40
30
20
10
0
BSA
huCD20
huCD20-BSA
msCD20
msCD20-BSA
KLH
ANTIGEN (20 ng)

FIG. 1A

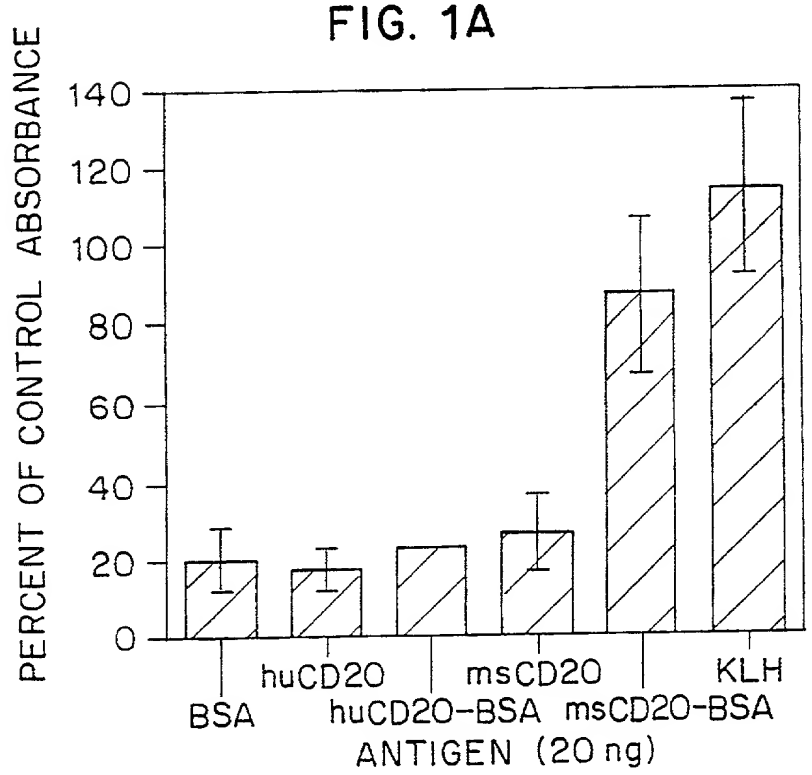
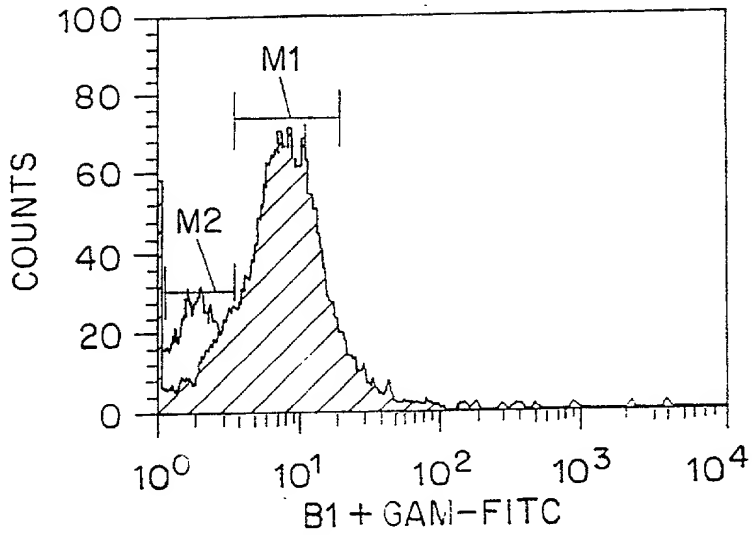

PERCENT OF CONTROL ABSORBANCE
140
120
100
80
60
40
20
0
BSA
huCD20
huCD20-BSA
msCD20
msCD20-BSA
KLH
ANTIGEN (20 ng)

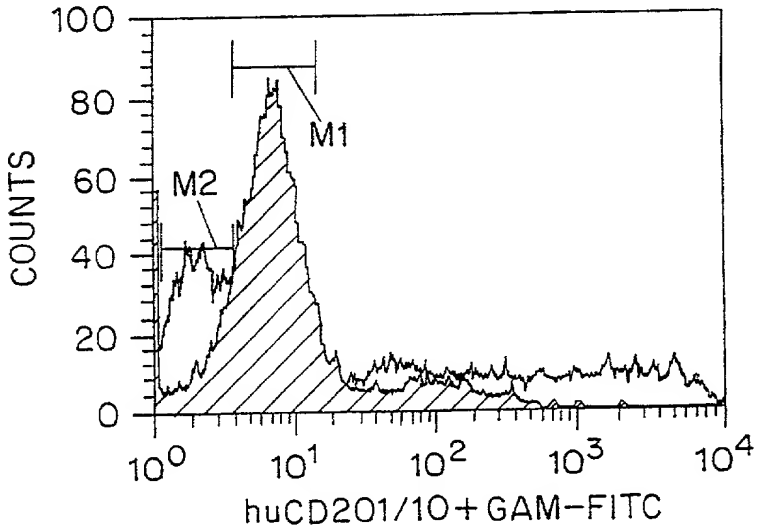
FIG. 1B



TOTAL EVENTS: 10000

MARKER	LEFT, RIGHT	EVENTS	% TOTAL
ALL	1, 9910	10000	100.00
M1	3, 17	7525	75.25
M2	1, 3	1201	12.01

FIG. 2A



TOTAL EVENTS: 10000

MARKER	LEFT, RIGHT	EVENTS	% TOTAL
ALL	1, 9910	10000	100.00
M1	3, 12	7327	73.27
M2	1, 4	1454	14.54

FIG. 2B

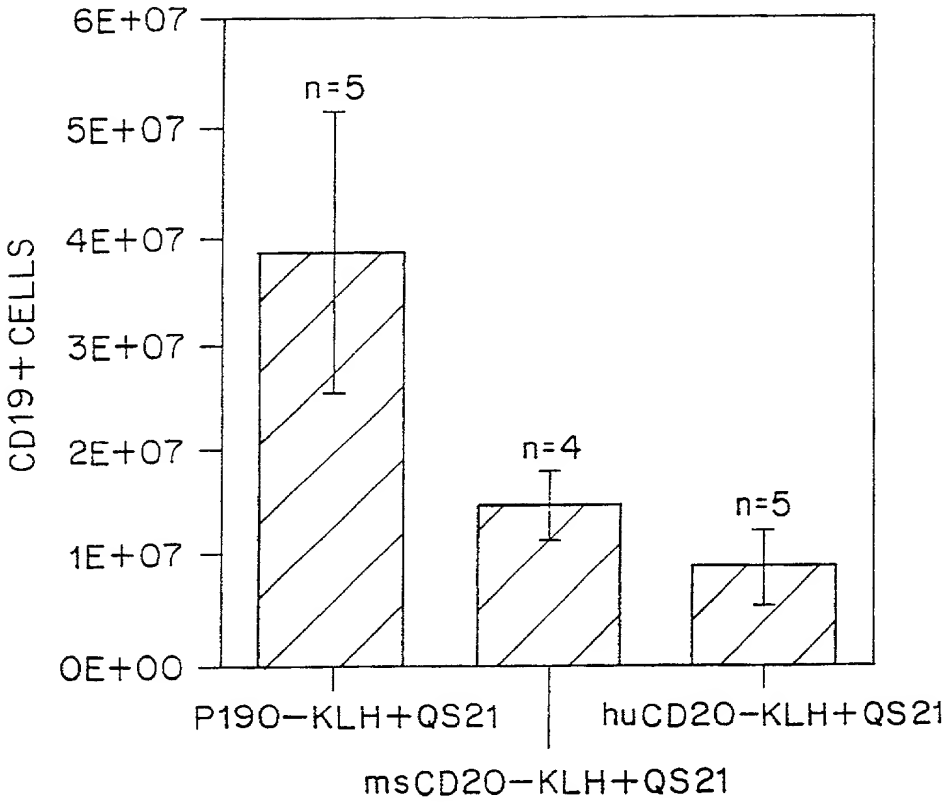

6E+07
5E+07
4E+07
3E+07
2E+07
1E+07
0E+00
CD19+CELLS
n=5
n=4
n=5
P190-KLH+QS21
huCD20-KLH+QS21
msCD20-KLH+QS21

FIG. 3

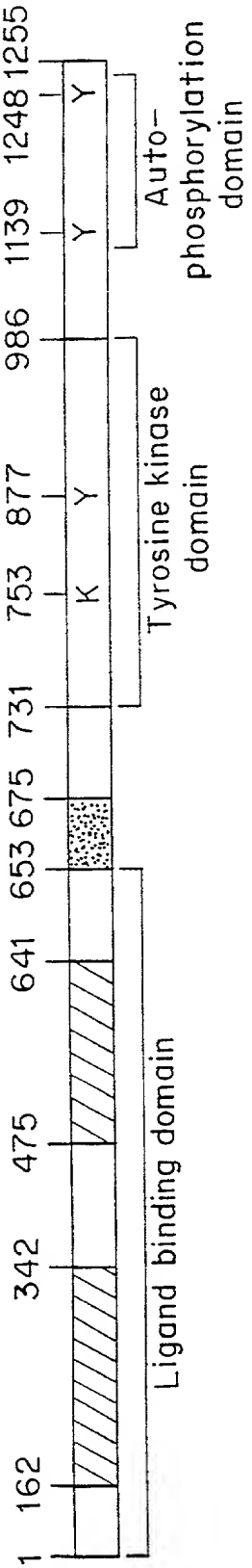

1
162
342
475
641
653
675
731
753
877
986
1139
1248
1255
Ligand binding domain
Tyrosine kinase domain
Auto-phosphorylation domain
K
Y
Y

FIG. 4

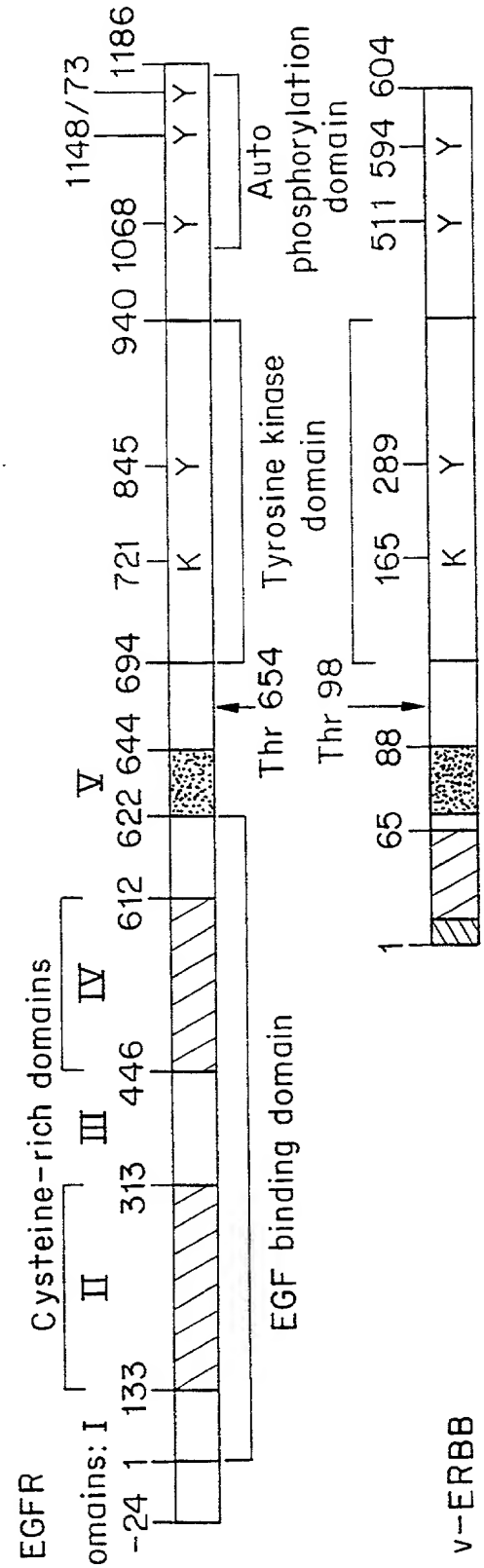

EGFR
Domains: I
-24
1
133
II
Cysteine-rich domains
313
III
446
IV
612
V
622
644
694
721
845
940
1068
1148/73
1186
EGF binding domain
Thr 654
Tyrosine kinase domain
Auto phosphorylation domain
K
Y
Y
Y
v-ERBB
1
65
88
Thr 98
165
289
511
594
604
K
Y
Y
Y

FIG. 5

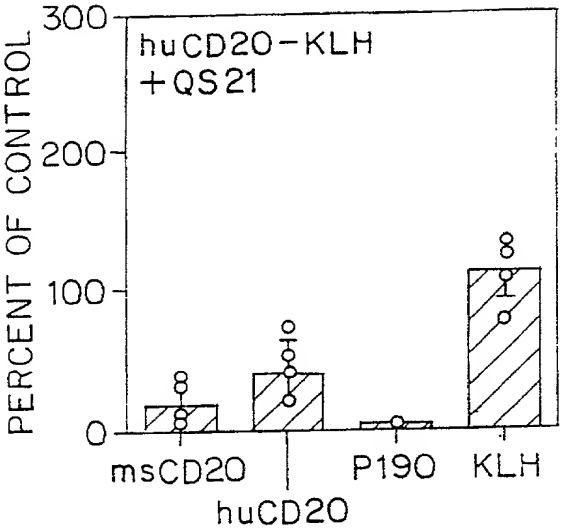


FIG. 6A

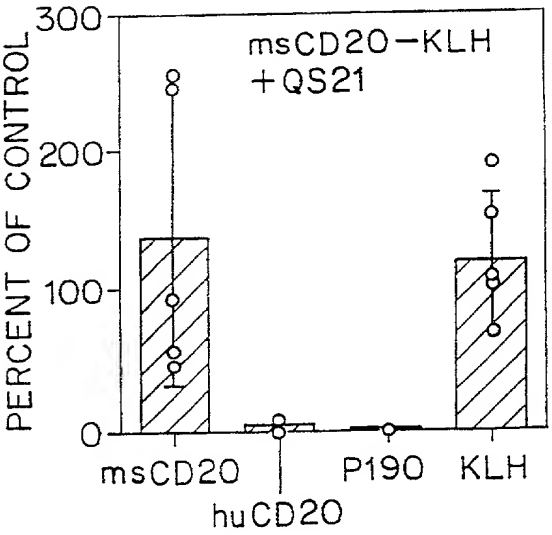


FIG. 6B

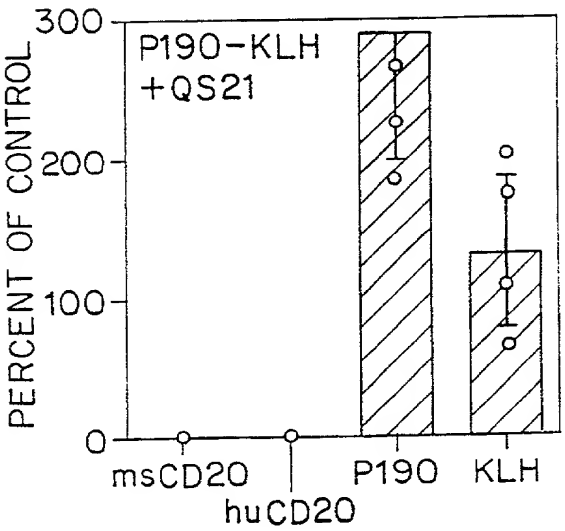


FIG. 6C

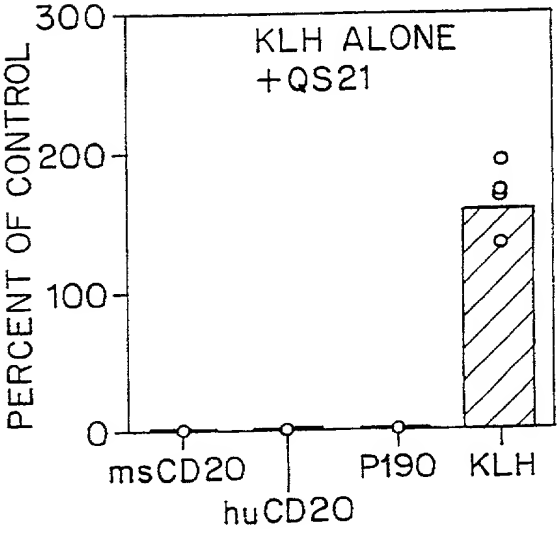


FIG. 6D

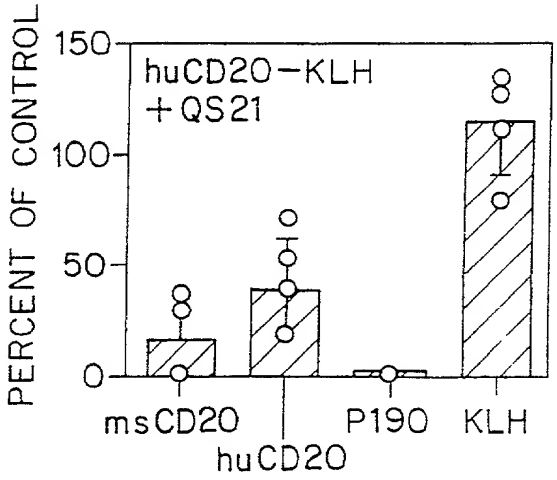


FIG. 7A

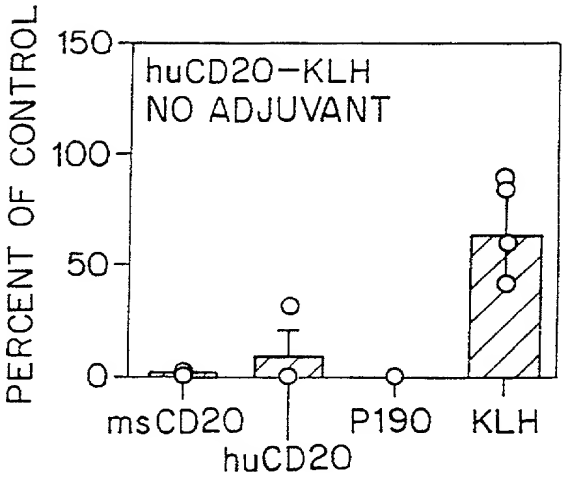


FIG. 7B

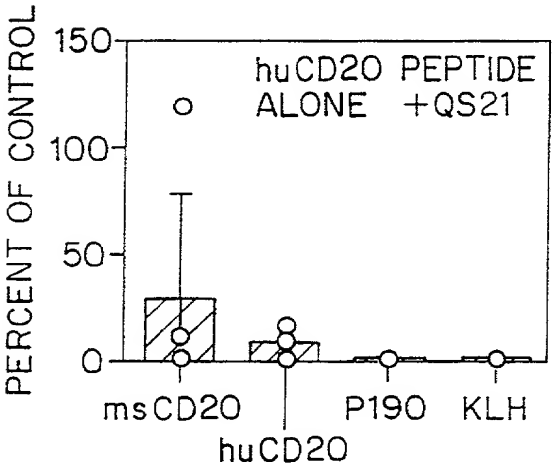


FIG. 7C

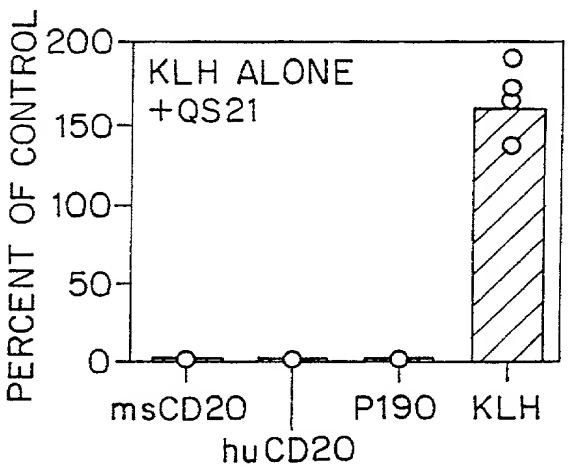


FIG. 7D

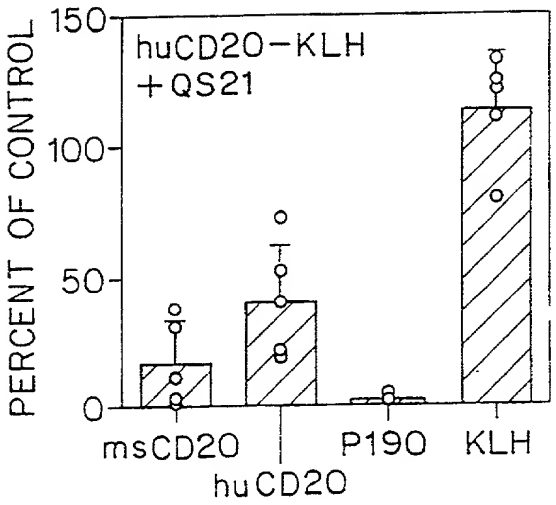


FIG. 8A

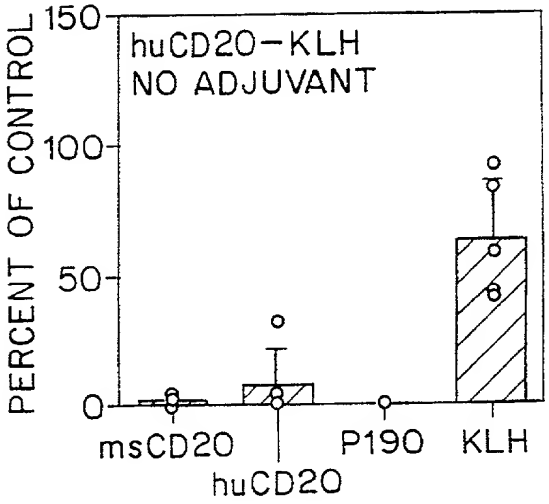


FIG. 8B

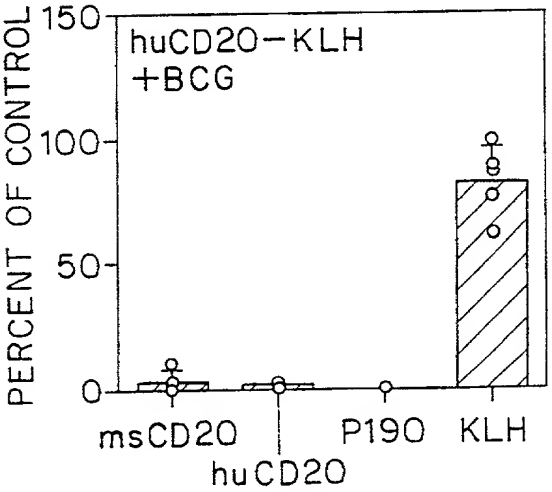


FIG. 8C

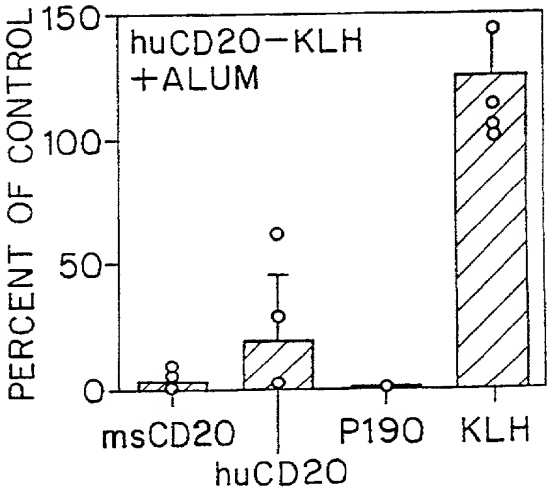


FIG. 8D

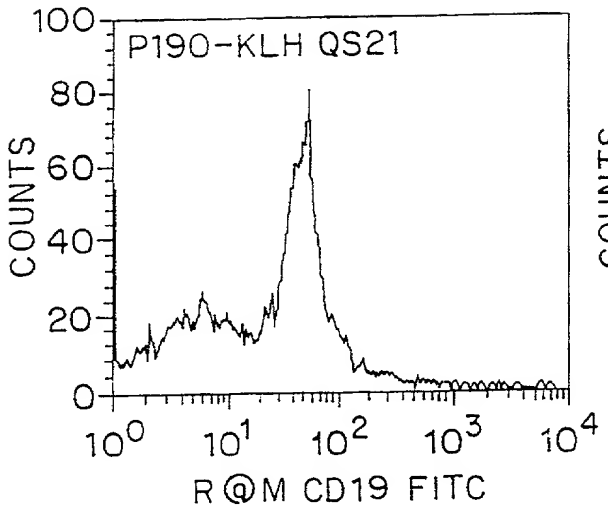


FIG. 9A

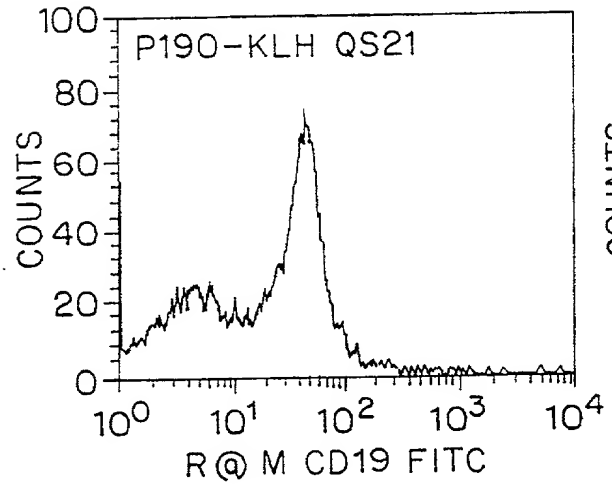


FIG. 9B

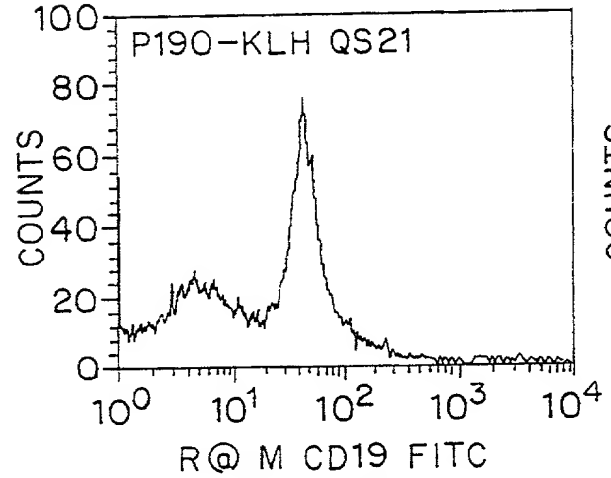


FIG. 9C

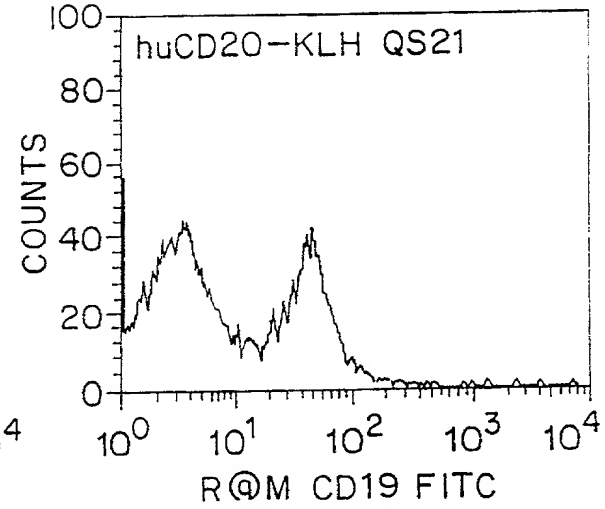


FIG. 9D

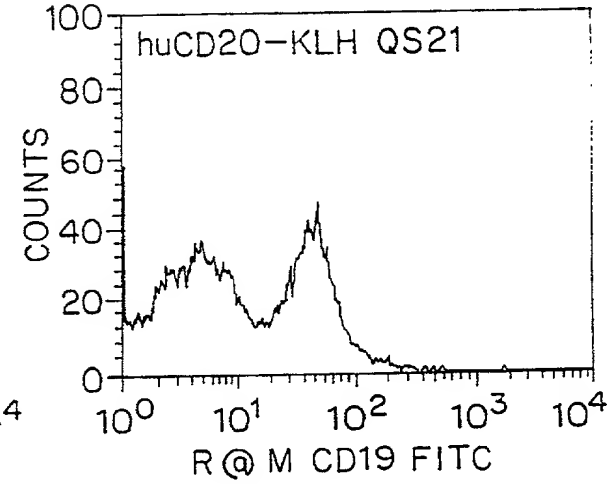


FIG. 9E

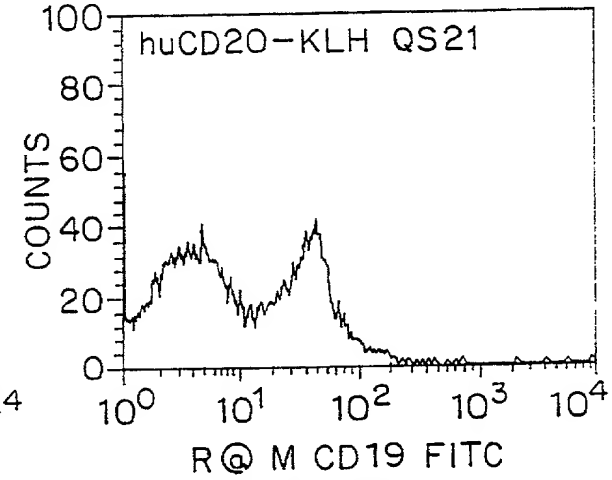


FIG. 9F

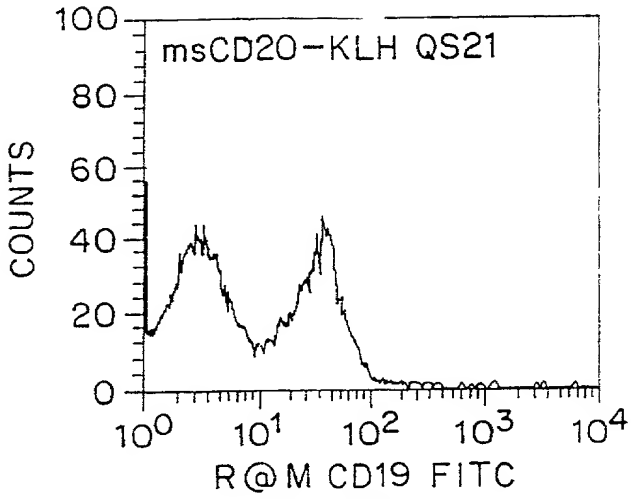


FIG. 9G

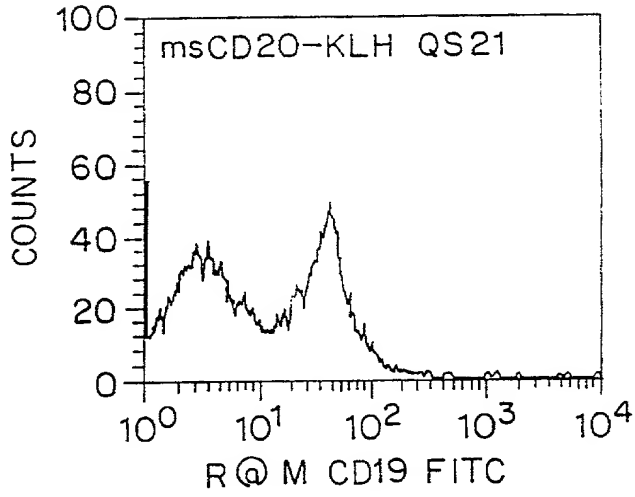


FIG. 9H

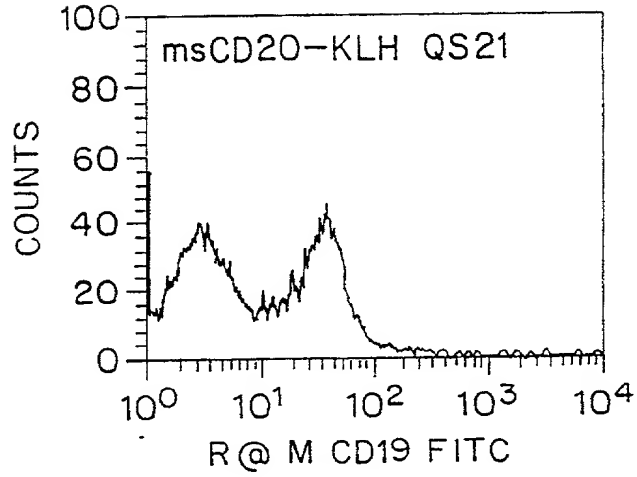


FIG. 9I

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My citizenship, residence and post office address are as listed below next to my name.

I believe I am the original, first and [ ] sole/[x] joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: Compositions and Methods for Active Vaccination

the specification of which

(a) [ ] is attached hereto.

(b) [ ] was filed on _____ as Application Serial No. _____ and was amended on _____.

(c) [X] was described and claimed in International Application No. PCT/US99/10065 filed on May 7, 1999 and amended on _____.

Acknowledgment of Duty of Disclosure

I hereby state that I have reviewed and understood the content of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of the subject matter claimed in this application in accordance with Title 37, Code of Federal Regulations § 1.56(a).

35 U.S.C. § 120

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose material information as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status)(patented,pending,abandoned)	(Patent No. if applicable)
(Application Serial No.)	(Filing Date)	(Status)(patented,pending,abandoned)	(Patent No. if applicable)

Power of Attorney

I hereby appoint Carl Oppedahl, PTO Reg. NO. 32,746, Marina T. Larson, PTO Reg. No. 32,038, and Nancy J. Parsons, PTO Reg. No. 40,364 of the firm of OPPEDAHL & LARSON LLP, having office at P.O. Box 5068, Alpine Bank Center, 2nd Floor, 256 Dillon Ridge Rd., Dillon, CO 80435 as attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

SEND CORRESPONDENCE TO:

021121

PATENT TRADEMARK OFFICE

DIRECT TELEPHONE CALLS TO:
OPPEDAHL & LARSON LLP
(970) 468-6600

OPPEDAHL & LARSON

FILE NO. MSKP039
INVENTOR . Agus, et al

Claim for Priority

I hereby claim foreign priority benefits under 35 U.S.C. § 119 (a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign applications for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

EARLIEST FOREIGN APPLICATION(S), FILED WITHIN TWELVE MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION					
COUNTRY	APPLICATION NO.	DATE OF FILING (day/month/year)	DATE OF ISSUE (day/month/year)	PRIORITY CLAIMED	CERTIFIED COPY ATTACHED
				YES[ ] NO[ ]	YES[ ] NO[ ]
FOREIGN APPLICATION(S), IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION					
COUNTRY	APPLICATION NO.	DATE OF FILING (day/month/year)	DATE OF ISSUE (day/month/year)		

Provisional Application

I hereby claim the benefit under 35 U.S.C § 119(e) of any United States provisional application(s) listed below.

60/084,870 — 08 May 1998
(application number) (filing date)

(application number) (filing date)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	AGUS	David	B.
RESIDENCE & CITIZENSHIP	CITY OF RESIDENCE ~~Brooklyn~~ Beverly Hills PA	STATE OR COUNTRY OF RESIDENCE ~~NY~~ CA PA	COUNTRY OF CITIZENSHIP US
POST OFFICE ADDRESS ~~9 Pierrepont Street~~ 522 North Crescent Drive CA PA	CITY ~~Brooklyn~~ Beverly Hills PA	STATE/COUNTRY ZIP CODE ~~NY 11021~~ CA 90210 PA	
DATE 11/1/00	SIGNATURE		

[X] Signature for additional joint inventor attached. Number of Pages 1.
[ ] Signature by Administrator(trix) or legal representative for deceased or incapacitated inventor. Number of Pages ___.
[ ] Signature for inventor who refuses to sign or cannot be reached by person authorized under 37 CFR § 1.47. Number of Pages ___.

NAME OF SECOND INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	SCHEINBERG	DAVID	A
RESIDENCE & CITIZENSHIP	**CITY OF RESIDENCE**	**STATE OR COUNTRY OF RESIDENCE**	**COUNTRY OF CITIZENSHIP**
	NEW YORK NY	NY	US
POST OFFICE ADDRESS		**CITY**	**STATE/COUNTRY ZIP CODE**
325 Central Park West		New York	NY 10025
DATE 11/1/00		**SIGNATURE**	

NAME OF THIRD INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	ROBERTS	WENDY	
RESIDENCE & CITIZENSHIP	**CITY OF RESIDENCE**	**STATE OR COUNTRY OF RESIDENCE**	**COUNTRY OF CITIZENSHIP**
	NEW YORK NY	NY	US
POST OFFICE ADDRESS		**CITY**	**STATE/COUNTRY ZIP CODE**
~~1233 York Avenue~~ 303 East 71 St		New York	NY 10021
DATE 11/1/00		**SIGNATURE**	

NAME OF FOURTH INVENTOR	LAST NAME	FIRST NAME	MIDDLE NAME
	ZELENETZ	ANDREW	D.
RESIDENCE & CITIZENSHIP	**CITY OF RESIDENCE**	**STATE OR COUNTRY OF RESIDENCE**	**COUNTRY OF CITIZENSHIP**
	LARCHMONT NY	NY	US
POST OFFICE ADDRESS		**CITY**	**STATE/COUNTRY ZIP CODE**
31 Mohegan Road		Larchmont	NY 10538
DATE 11/6/2000		**SIGNATURE**	

OPPEDAHL & LARSON

FILE NO. MSKP039
INVENTOR . Agus, et al

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My citizenship, residence and post office address are as listed below next to my name.

I believe I am the original, first and [ ] sole/[x] joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: Compositions and Methods for Active Vaccination

the specification of which

(a) [ ] is attached hereto.

(b) [ ] was filed on ______________ as Application Serial No. ____________ and was amended on __________.

(c) [X] was described and claimed in International Application No. PCT/US99/10065 filed on May 7, 1999 and amended on __________.

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(Application Serial No.)	(Filing Date)	(Status)(patented,pending,abandoned)	(Patent No. if applicable)

(Application Serial No.)	(Filing Date)	(Status)(patented,pending,abandoned)	(Patent No. if applicable)

Power of Attorney

I hereby appoint Carl Oppedahl, PTO Reg. NO. 32,746, Marina T. Larson, PTO Reg. No. 32,038, and Nancy J. Parsons, PTO Reg. No. 40,364 of the firm of OPPEDAHL & LARSON LLP, having office at P.O. Box 5068, Alpine Bank Center, 2nd Floor, 256 Dillon Ridge Rd., Dillon, CO 80435 as attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

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EARLIEST FOREIGN APPLICATION(S), FILED WITHIN TWELVE MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION					
COUNTRY	APPLICATION NO.	DATE OF FILING (day/month/year)	DATE OF ISSUE (day/month/year)	PRIORITY CLAIMED	CERTIFIED COPY ATTACHED
				YES[ ] NO[ ]	YES[ ] NO[ ]
FOREIGN APPLICATION(S), IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION					
COUNTRY	APPLICATION NO.	DATE OF FILING (day/month/year)	DATE OF ISSUE (day/month/year)		

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60/084,870 — 08 May 1998
(application number) (filing date)

(application number) (filing date)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

NAME OF SOLE OR FIRST INVENTOR	LAST NAME AGUS	FIRST NAME David	MIDDLE NAME B.
RESIDENCE & CITIZENSHIP	CITY OF RESIDENCE Brooklyn	STATE OR COUNTRY OF RESIDENCE NY	COUNTRY OF CITIZENSHIP US
POST OFFICE ADDRESS 9 Pierrepont Street		CITY Brooklyn	STATE/COUNTRY ZIP CODE NY 10021
DATE		SIGNATURE	

[X] Signature for additional joint inventor attached. Number of Pages 1.
[ ] Signature by Administrator(trix) or legal representative for deceased or incapacitated inventor. Number of Pages ___.
[ ] Signature for inventor who refuses to sign or cannot be reached by person authorized under 37 CFR § 1.47. Number of Pages ___.

OPPEDAHL & LARSON

FILE NO. MSKP039
INVENTOR . Agus, et al

NAME OF SECOND INVENTOR	LAST NAME SCHEINBERG	FIRST NAME DAVID	MIDDLE NAME
RESIDENCE & CITIZENSHIP	CITY OF RESIDENCE NEW YORK	STATE OR COUNTRY OF RESIDENCE NY	COUNTRY OF CITIZENSHIP US
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DATE		SIGNATURE	

NAME OF THIRD INVENTOR	LAST NAME ROBERTS	FIRST NAME WENDY	MIDDLE NAME
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DATE		SIGNATURE	

NAME OF FOURTH INVENTOR	LAST NAME ZELENETZ	FIRST NAME ANDREW	MIDDLE NAME D.
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DATE		SIGNATURE	

SEQUENCE LISTING

<110> Agus, David B.
Scheinberg, David
Zelenetz, Andrew D.
Roberts, Wendy

<120> Compositions and Methods for Active Vaccination

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His Phe Leu Lys His Glu Ser Leu Asn Phe Ile Arg Ala His Thr Pro
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35 40 45

Leu Tyr Gln Gly Cys Gln Val Val Gln Gly Asn Leu Glu Leu Thr Tyr
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Leu Pro Thr Asn Ala Ser Leu Ser Phe Leu Gln Asp Ile Gln Glu Val
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Gln Gly Tyr Val Leu Ile Ala His Asn Gln Val Arg Gln Val Pro Leu
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Gln Arg Leu Arg Ile Val Arg Gly Thr Gln Leu Phe Glu Asp Asn Tyr
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Ala Leu Ala Val Leu Asp Asn Gly Asp Pro Leu Asn Asn Thr Thr Pro
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Val Thr Gly Ala Ser Pro Gly Gly Leu Arg Glu Leu Gln Leu Arg Ser
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Leu Thr Glu Ile Leu Lys Gly Gly Val Leu Ile Gln Arg Asn Pro Gln
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Leu Cys Tyr Gln Asp Thr Ile Leu Trp Lys Asp Ile Phe His Lys Asn
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Asn Gln Leu Ala Leu Thr Leu Ile Asp Thr Asn Arg Ser Arg Ala Cys
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His Pro Cys Ser Pro Met Cys Lys Gly Ser Arg Cys Trp Gly Glu Ser

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Ser Glu Asp Cys Gln Ser Leu Thr Arg Thr Val Cys Ala Gly Gly Cys
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Ala Arg Cys Lys Gly Pro Leu Pro Thr Asp Cys Cys His Glu Gln Cys
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Ala Ala Gly Cys Thr Gly Pro Lys His Ser Asp Cys Leu Ala Cys Leu
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His Phe Asn His Ser Gly Ile Cys Glu Leu His Cys Pro Ala Leu Val
260 265 270

Thr Tyr Asn Thr Asp Thr Phe Glu Ser Met Pro Asn Pro Glu Gly Arg
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Tyr Thr Phe Gly Ala Ser Cys Val Thr Ala Cys Pro Tyr Asn Tyr Leu
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Ser Thr Asp Val Gly Ser Cys Thr Leu Val Cys Pro Leu His Asn Gln
305 310 315 320

Glu Val Thr Ala Glu Asp Gly Thr Gln Arg Cys Glu Lys Cys Ser Lys
325 330 335

Pro Cys Ala Arg Val Cys Tyr Gly Leu Gly Met Glu His Leu Arg Glu
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Val Arg Ala Val Thr Ser Ala Asn Ile Gln Glu Phe Ala Gly Cys Lys
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Lys Ile Phe Gly Ser Leu Ala Phe Leu Pro Glu Ser Phe Asp Gly Asp
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385 390 395 400

Glu Thr Leu Glu Glu Ile Thr Gly Tyr Leu Tyr Ile Ser Ala Trp Pro
405 410 415

Asp Ser Leu Pro Asp Leu Ser Val Phe Gln Asn Leu Gln Val Ile Arg
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Gln Leu Cys Ala Arg Gly His Cys Trp Gly Pro Gly Pro Thr Gln Cys
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Ser Tyr Met Pro Ile Trp Lys Phe Pro Asp Glu Glu Gly Ala Cys Gln
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Pro Cys Pro Ile Asn Cys Thr His Ser Cys Val Asp Leu Asp Asp Lys
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Gly Cys Pro Ala Glu Gln Arg Ala Ser Pro Leu Thr Ser Ile Ile Ser
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Ala Val Val Gly Ile Leu Leu Val Val Val Leu Gly Val Val Phe Gly
660 665 670

Ile Leu Ile Lys Arg Arg Gln Gln Lys Ile Arg Lys Tyr Thr Met Arg
675 680 685

Arg Leu Leu Gln Glu Thr Glu Leu Val Glu Pro Leu Thr Pro Ser Gly
690 695 700

Ala Met Pro Asn Gln Ala Gln Met Arg Ile Leu Lys Glu Thr Glu Leu

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725 730 735

Gly Ile Trp Ile Pro Asp Gly Glu Asn Val Lys Ile Pro Val Ala Ile
740 745 750

Lys Val Leu Arg Glu Asn Thr Ser Pro Lys Ala Asn Lys Glu Ile Leu
755 760 765

Asp Glu Ala Tyr Val Met Ala Gly Val Gly Ser Pro Tyr Val Ser Arg
770 775 780

Leu Leu Gly Ile Cys Leu Thr Ser Thr Val Gln Leu Val Thr Gln Leu
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Met Pro Tyr Gly Cys Leu Leu Asp His Val Arg Glu Asn Arg Gly Arg
805 810 815

Leu Gly Ser Gln Asp Leu Leu Asn Trp Cys Met Gln Ile Ala Lys Gly
820 825 830

Met Ser Tyr Leu Glu Asp Val Arg Leu Val His Arg Asp Leu Ala Ala
835 840 845

Arg Asn Val Leu Val Lys Ser Pro Asn His Val Lys Ile Thr Asp Phe
850 855 860

Gly Leu Ala Arg Leu Leu Asp Ile Asp Glu Thr Glu Tyr His Ala Asp
865 870 875 880

Gly Gly Lys Val Pro Ile Lys Trp Met Ala Leu Glu Ser Ile Leu Arg
885 890 895

Arg Arg Phe Thr His Gln Ser Asp Val Trp Ser Tyr Gly Val Thr Val
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Trp Glu Leu Met Thr Phe Gly Ala Lys Pro Tyr Asp Gly Ile Pro Ala
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Arg Glu Ile Pro Asp Leu Leu Glu Lys Gly Glu Arg Leu Pro Gln Pro
930 935 940

Pro Ile Cys Thr Ile Asp Val Tyr Met Ile Met Val Lys Cys Trp Met
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Ile Asp Ser Glu Cys Arg Pro Arg Phe Arg Glu Leu Val Ser Glu Phe

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Ser Arg Met Ala Arg Asp Pro Gln Arg Phe Val Val Ile Gln Asn Glu
980 985 990

Asp Leu Gly Pro Ala Ser Pro Leu Asp Ser Thr Phe Tyr Arg Ser Leu
995 1000 1005

Leu Glu Asp Asp Asp Met Gly Asp Leu Val Asp Ala Glu Glu Tyr Leu
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Val Pro Gln Gln Gly Phe Phe Cys Pro Asp Pro Ala Pro Gly Ala Gly
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Asp Leu Gly Met Gly Ala Ala Lys Gly Leu Gln Ser Leu Pro Thr His
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1155 1160 1165

Arg Pro Lys Thr Leu Ser Pro Gly Lys Asn Gly Val Val Lys Asp Val
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Pro Pro Ser Thr Phe Lys Gly Thr Pro Thr Ala Glu Asn Pro Glu Tyr
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35 40 45

Leu Ser Leu Gln Arg Met Phe Asn Asn Cys Glu Val Val Leu Gly Asn
50 55 60

Leu Glu Ile Thr Tyr Val Gln Arg Asn Tyr Asp Leu Ser Phe Leu Lys
65 70 75 80

Thr Ile Gln Glu Val Ala Gly Tyr Val Leu Ile Ala Leu Asn Thr Val
85 90 95

Glu Arg Ile Pro Leu Glu Asn Leu Gln Ile Ile Arg Gly Asn Met Tyr
100 105 110

Tyr Glu Asn Ser Tyr Ala Leu Ala Val Leu Ser Asn Tyr Asp Ala Asn
115 120 125

Lys Thr Gly Leu Lys Glu Leu Pro Met Arg Asn Leu Gln Glu Ile Leu
130 135 140

His Gly Ala Val Arg Phe Ser Asn Asn Pro Ala Leu Cys Asn Val Glu
145 150 155 160

Ser Ile Gln Trp Arg Asp Ile Val Ser Ser Asp Phe Leu Ser Asn Met
165 170 175

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180 185 190

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210 215 220

Gly Lys Ser Pro Ser Asp Cys Cys His Asn Gln Cys Ala Ala Gly Cys
225 230 235 240

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